Characterization of a Murine Model of *Ureaplasma urealyticum* Pneumonia

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Ureaplasma urealyticum respiratory tract colonization in preterm infants has been associated with a high incidence of pneumonia and the development of bronchopulmonary dysplasia. However, study of this human pathogen has been hampered by the absence of animal models. We have developed the first juvenile mouse model of *Ureaplasma* pneumonia and characterized the histopathology during the month following inoculation. C3H/HeN mice were inoculated intratracheally with a mouse-adapted clinical *Ureaplasma* isolate (biovar 2) or sham inoculated with 10B broth. Culture of lung homogenates and PCR of DNA from bronchoalveolar lavage fluid (BAL) confirmed the presence of *Ureaplasma* in 100% of inoculated animals at 1 day, 60% at 2 days, 50% at 3 days, and 25% at 7 and 14 days. *Ureaplasma* was undetectable 28 days postinoculation. There were marked changes in BAL and interstitial-cell composition with increased number of polymorphonuclear leukocytes 1 to 2 days and 14 days postinoculation and macrophages at 2 and 14 days postinoculation. The *Ureaplasma* infection caused a persistent focal loss of airway ciliated epithelium and a mild increase in interstitial cellularity. There were no differences in BAL protein concentration during the first 28 days, suggesting that pulmonary vascular endothelial barrier integrity remained intact. Comparison of BAL cytokine and chemokine concentrations revealed low levels of tumor necrosis factor alpha (TNF-α) at 3 days and monocyte chemoattractant protein 1 at 7 days in *Ureaplasma*-infected mice but a trend toward increased TNF-α at 14 days and increased granulocyte-macrophage colony-stimulating factor and interleukin-10 at 28 days. These data suggest that *Ureaplasma* alone may cause limited inflammation and minimal tissue injury in the early phase of infection but may promote a mild chronic inflammatory response in the later phase of infection (days 14 to 28), similar to the process that occurs in human newborns.

Ureaplasma urealyticum is a mucosal pathogen of humans that lacks a cell wall and that is subtyped into two biovars and 14 serovars (24). Based on significant genotypic differences between the two biovars, it has been proposed to separate the species previously known as *U. urealyticum* into two new species, *Ureaplasma parvum* (biovar 1 containing serovars 1, 3, 6, and 14) and *Ureaplasma urealyticum* (biovar 2 containing serovars 2, 4, 5, and 7 to 13) (23, 24). Because *Ureaplasma* is a commensal in the adult female genital tract, it has been considered of low virulence. However, *Ureaplasma* is the most common organism isolated from women with obstetric complications (15, 38, 48). *Ureaplasma* has been isolated from blood, cerebrospinal fluid, tracheal aspirates, and lung tissue of newborn infants (30, 44). Respiratory tract colonization in preterm infants has been associated with a high incidence of pneumonia (10, 32), severe respiratory failure and death (30), and the development of bronchopulmonary dysplasia (BPD) (1, 35, 47). Some serovars have been implicated in adverse pregnancy outcomes more commonly than others (2, 16, 19, 49). Although *U. parvum* is more commonly isolated from clinical specimens (2, 19), there is a higher rate of BPD in

U. urealyticum-colonized infants (2). This suggests that the latter biovar may be more virulent.

Recent studies suggest that *Ureaplasma* may contribute to neonatal lung injury by augmenting the pulmonary inflammatory response. Preterm intubated infants who were colonized with *Ureaplasma* had higher tracheal aspirate interleukin-1β (IL-1β) concentrations and neutrophil chemotactic activity on the first day of life than noncolonized infants (17). We previously observed that *Ureaplasma* respiratory tract colonization in infants of ≤1,250 g birth weight was associated with increases in tracheal aspirate concentrations of proinflammatory cytokines tumor necrosis factor alpha (TNF-α), IL-1β, and IL-8 relative to counterregulatory cytokine IL-6 in the first week of life (34). The stimulatory effect of *Ureaplasma* on cytokine release has been confirmed in vitro. We previously reported that *Ureaplasma* alone and in combination with bacterial endotoxin (lipopolysaccharide [LPS]) induced concentration- and development-dependent changes in cytokine release by cultured monocytes from preterm and full-term newborns and adults (27). In that study, in vitro inoculation with low-inoculum *Ureaplasma* (10^3 color changing units [CCU]) (i) stimulated TNF-α and IL-8 release by preterm cells, (ii) augmented LPS-stimulated TNF release in all cells, and (iii) partially blocked LPS-stimulated IL-6 release by all cells and reduced LPS-stimulated IL-10 release by preterm cells. These data suggest that *Ureaplasma* might enhance the proinflammatory response to a second infection by stimulating proinflammatory cytokine (TNF-α and

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IL-8) expression and blocking counterregulatory cytokine (IL-6 and IL-10) expression, thereby predisposing the preterm infant to prolonged, dysregulated inflammation, lung injury, and impaired clearance of secondary infections.

Previously, experimental animal models have been limited to preterm baboons (46) and newborn mice (39). The former model is limited by its high cost, and the latter is limited by the technical demands and lack of available tissue due to the size of neonatal mice. To facilitate further analysis of the host response to *Ureaplasma* infection, we have developed the first murine model of *Ureaplasma* pneumonia in juvenile mice. In the present study, we describe the changes in cell composition of bronchoalveoalar lavage fluid (BAL), parenchymal inflammation, indices of injury, and lung histology during 4 weeks after inoculation with a clinical isolate of *U. urealyticum* (biovar 2).

MATERIALS AND METHODS

Animals. Eight-week-old *Mycoplasma pulmonis*-free CD-1 outbred mice and C3H/HeN mice were obtained from Charles River (Wilmington, Mass.) and were housed in the Central Animal Facility of the University of Maryland at Baltimore in autoclaved microisolation cages with autoclaved bedding by using standard barrier techniques. The protocol was approved by the University of Maryland Institutional Animal Care and Use Committee.

Inoculation. In initial experiments groups of three CD-1 outbred and C3H/HeN mice were inoculated intratracheally with 10^6 CCU of either American Type Culture Collection (ATCC; Manassas, Va.) serotype 3 reference strain or a clinical isolate from a human preterm infant with BPD identified as *U. urealyticum* (biovar 2) by biovar-specific primers (24). Twenty-four hours after inoculation the lungs were harvested, homogenized, and cultured in 10B broth. Despite repeated attempts, no viable organisms were recovered from mice of either strain inoculated with the ATCC serotype 3 reference strain or CD-1 mice inoculated with the clinical isolate. Two-thirds of C3H/HeN mice inoculated with the clinical isolate were culture positive after 24 h. Recovered *Ureaplasma* was passaged in media and passaged again in C3H/HeN mice sacrificed 24, 48, and 72 h postinoculation. *Ureaplasma* recovered from one mouse at 72 h (mouse-adapted isolate) was grown to late log phase in 10B medium (40) and aliquoted at 10^7 CCU/ml (37) and frozen at $-70°C$. Prior to intratracheal inoculation, mice were anesthetized with 10 mg of xylazine/kg of body weight and 65 mg of ketamine/kg intraperitoneally. Each mouse was inoculated intratracheally with 10^6 CCU of the mouse-adapted *U. urealyticum* isolate in 100 μl of 10B broth by instilling the inoculum into the posterior pharynx of an anesthetized mouse while it was suspended in a vertical position and prevented from swallowing by gentle extension of the tongue. Control mice received 10B broth alone. The mice were maintained in this position until aspiration was witnessed (disappearance of inoculum from the posterior pharynx and retraction of the chest wall). Groups of four mice were sacrificed 1, 2, 3, 7, 14, and 28 days postinoculation for bronchoalveolar lavage and analysis of lung tissue for *Ureaplasma* by culture and PCR. Additional groups of four mice were sacrificed at the same time points, and lungs were collected for histology and immunohistochemical analysis. Untreated mice were sacrificed at a single time point.

Bronchoalveolar lavage. Bronchoalveolar lavage was performed in situ through a 21-gauge blunt-end needle secured in the trachea by using 1 ml of phosphate-buffered saline (PBS) instilled and withdrawn twice, followed by instillation and recovery of a second 1-ml aliquot of PBS. BAL was stored at 4°C until cells were collected by centrifugation at $1,000 \times g$ for 3 min. Cell-free supernatants were stored at $-80°C$ for analysis of total-protein and cytokine concentrations. Total-cell counts were performed manually with a hemacytometer, and differential cell counts of Diff-Quick-stained cytopreparations were performed by two blinded observers (J.K. and R.M.V.) using morphological criteria. BAL protein was measured by the Bradford method (5).

Cytokine ELISA. A broad selection of cytokines and chemokines that have been associated with lung injury (11, 12, 31, 42) and/or the development of BPD (3, 4, 22, 25, 33) were selected for study. Murine TNF-α, major inflammatory protein 2 (MIP-2), the mouse analogue of human growth-related protein alpha (KC), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP-1), IL-6, IL-10, and IL-11 concentrations were measured in the UMB Cytokine Core Laboratory by using standard two-antibody enzyme-linked immunosorbent assays (ELISA) with commercial antibody pairs and recombinant standards (TNF-α from Biosource, Camarillo, Calif.;

IL-6, IL-10, and GM-CSF from Pierce/Endogen, Woburn, Mass.; KC, MCP-1, MIP-2, and IL-11 from R & D Systems, Minneapolis, Minn.) as previously described (21). A curve was fit to the standards with a computer program (Softpro; Molecular Devices), and cytokine concentrations from each sample were calculated from the standard curve. Samples were analyzed in duplicate. Lower limits of detection were 3.9, 62.5,15.6, 3.125,15.6, 3.1, 7.8, and 31.2 pg/ml for TNF-α, MIP-2, KC, GM-CSF, MCP-1, IL-6, IL-10, and IL-11, respectively.

Lung culture. Following lavage the lungs were removed and processed for *Ureaplasma* culture. The lungs were homogenized between the frosted ends of sterile glass slides and placed in 10B broth. After a 24-h inoculation, 200 μl of the lung culture was added to 2 ml of fresh 10B broth. The tubes were incubated at 37°C in 95% air–5% CO_2. If color change occurred, 0.3 ml of inoculum was plated on A7 agar (Northeast Laboratory, Waterville, Maine). Tube cultures and plates were examined daily for 2 weeks for color change and typical colonies of *Ureaplasma*, respectively.

PCR. DNA was extracted from BAL with Qiagen columns according to the manufacturer's protocol (Qiagen, Valencia, Calif.). Each 50 μl of reaction mixture contained 20 mM Tris (pH 8.4); 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM (each) dATP, dGTP, dTTP, and dCTP; 2.5 U of *Taq* polymerase (Platinum PCR supermix; Gibco-BRL, Grand Island, N.Y.); 10 μl of sample, and 20 pmol each of primers UMS 170 (GTA TTT GCA ATC TTT ATA TGT TTT CG) and UMA 263 (TTT GTT GTT GCG TTT TCT G) (synthesized by Gibco-BRL) (24). The reaction mixtures were subjected to the following thermal cycling parameters in the Robocycler 40 (Stratagene): 1 cycle of 95°C for 5 min; 40 cycles of 95°C for 45 s, 54°C for 45 s, and 72°C for 45 s; and 1 cycle of 72°C for 7 min. Positive (100 CCU of *U. urealyticum* serotype 8; ATCC) and negative (sterile water) controls were processed in parallel with the test samples to detect false negatives and contamination, respectively. PCR products (10 μl) were analyzed by electrophoresis in 1.5% agarose gels stained with 0.5 μg of ethidium bromide/ml. A DNA product band of 476 bp visualized by UV illumination was considered positive.

Histology. Animals were sacrificed by CO_2 narcosis and cervical dislocation. The anterior chest wall was removed, the trachea was cannulated with a 21-gauge blunt needle, and the lungs were inflated in situ with 10% buffered formalin at 20 cm of H_2O pressure. The trachea and both lungs were removed en bloc and fixed in formalin. The formalin-fixed sections were analyzed for inflammation and injury by a blinded observer (S.R.). For immunohistologic analysis, lungs were harvested for frozen sectioning by inflation with a fixed 0.7-ml volume of 50% OCT diluted 1:1 with PBS. The lungs were embedded in undiluted OCT, and 8-μm-thick cryosections were cut and fixed with acetone for 3 min at $-20°C$ and stored at $-80°C$ for immunostaining. Slides were washed in PBS at 25°C for 20 min. Endogenous peroxidase was inactivated by incubation for 10 min with 30% hydrogen peroxide in methanol at 25°C. Nonspecific signal was reduced by sequential blocking with 5% (vol/vol) serum in PBS for 30 min and then with a commercial avidin-biotin blocking kit (Vector; Burlingame, Calif.) according to the manufacturer's instructions. The blocked slides were sequentially incubated with the primary antibody for 1 h, a secondary antibody (in some cases) for 30 min, a commercial avidin-biotin peroxidase detection system (Vector) according to the manufacturer's instructions, and 1 mg of diaminobenzidine (Sigma) in 0.02% hydrogen peroxide in PBS. All blocking and subsequent incubations were at 25°C. The blocking serum, primary, and secondary antibodies used for each cell type are as follows. For macrophages, the serum was mouse serum, the primary antibody was 1 μg of rat anti-mouse Mac-3/ml, and the secondary antibody was 2.5 μg of biotinylated goat anti-rat immunoglobulin G1 and G2a (both from BD-Pharmingen, San Diego, Calif.). For polymorphonuclear leukocytes (PMN), the serum was bovine serum, and the primary antibody was 2.5 μg of biotinylated rat anti-mouse Gr-1 (BD-Pharmingen)/ml. Immunostained slides were counterstained with hematoxylin as described in the manufacturer's protocol (BD Sciences) and were scored by a blinded observer (R.M.V.) by counting the number of positively stained cells in each high-power field (hpf). Four fields from comparable regions were counted in each section.

Statistical analysis. All data are presented as means ± standard errors (SE). Differences among experimental and sham-treated and untreated control groups at each time point and within-group comparisons over time were tested by a Fisher protected least-squares difference applied to a one-way analysis of variance. A P value of <0.05 was considered significant. Since there were no differences between untreated and broth-inoculated controls, only the differences between the experimental and broth-inoculated controls are shown in the figures.

RESULTS

***U. urealyticum* clearance.** None of the *Ureaplasma*-inoculated mice appeared ill, and there was no mortality. Culture of
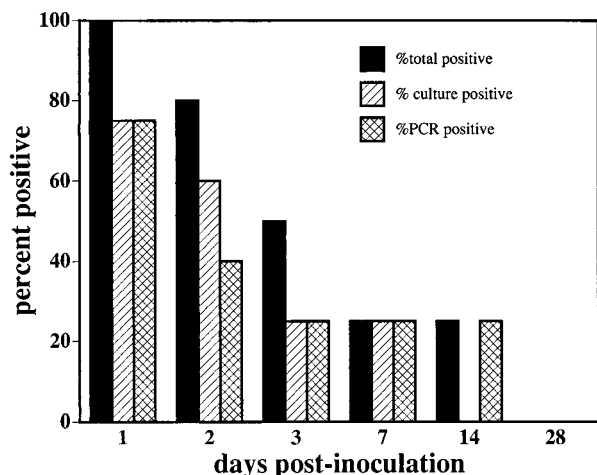
FIG. 1. *U. urealyticum* clearance from inoculated mice. Lungs of *Ureaplasma*-inoculated mice were homogenized and cultured in 10B broth, and PCR of BAL DNA was performed. Data are expressed as the percentages of animals who were *Ureaplasma* positive by one or both methods (black bars), lung culture positive (hatched bars), or BAL PCR positive (cross-hatched bars) on each day postinoculation
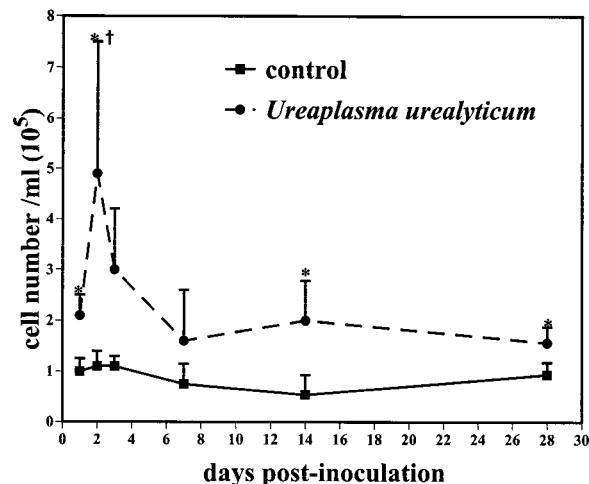

FIG. 2. BAL inflammatory cell number after *U. urealyticum* inoculation. Lungs of *Ureaplasma*-inoculated animals were lavaged in situ with two 1-ml aliquots of PBS. Total-cell counts were performed manually with a hemacytometer. Data are expressed as the means ± SE of the numbers of cells per milliliter of BAL in broth-inoculated controls (solid squares) and *Ureaplasma*-inoculated mice (solid circles) from 1 to 28 days postinoculation. ∗, $P < 0.05$ compared to controls of the same day; †, $P < 0.05$ compared to *Ureaplasma* group at all other time points.

lung tissue and PCR of DNA from BAL confirmed the presence of *Ureaplasma* in 100% of inoculated animals at 24 h, 60% at 48 h, 50% at 72 h, 25% at 7 and 14 days, and 0% at 28 days (Fig. 1). *Ureaplasma* was detected by PCR, but not by lung culture, in 25% of inoculated mice at 24 h, 48 h, 72 h, and 14 days, suggesting that detected *Ureaplasma* cells in these animals were nonviable organisms. All lungs of broth-inoculated and untreated mice were culture and PCR negative.

U. urealyticum infection stimulated a neutrophil and macrophage influx into the lungs. *Ureaplasma* inoculation stimulated significant changes in BAL cell composition characterized by an influx of PMN and macrophages. Total BAL cell counts were 2-, 4.4-, 3.7-, and 1.7-fold higher at 1, 2, 14, and 28 days postinoculation (all $P < 0.05$), respectively, in the *Ureaplasma*-inoculated mice than in the broth-inoculated controls (Fig. 2). Two peaks of BAL PMN accumulation occurred at 24 to 48 h (9.7- and 11-fold-higher numbers of PMN in *Ureaplasma*-inoculated mice than in controls) and 14 days postinoculation (23-fold-higher numbers of PMN in *Ureaplasma*-inoculated mice than in controls) (Fig. 3A). BAL macrophage content of *Ureaplasma*-inoculated mice paralleled PMN content, increasing to 3.8 and 2.7 times control levels at 2 and 14 days postinoculation, respectively (Fig. 3B).

Immunohistochemistry with cell-specific antibodies revealed 2.1-, 2.3-, and 4.5-fold-higher numbers of PMN/hpf in lungs of *Ureaplasma*-inoculated mice than in broth-inoculated controls at 2, 14, and 28 days, respectively (all $P < 0.05$) (Fig. 4A). By 24 to 48 h postinoculation, PMN increased throughout the lung interstitium and accumulated within focal areas of consolidation (Fig. 4B). The number of macrophages per hpf were 4.5-, 2-, 2.6-, and 2.6-fold higher in the lungs of *Ureaplasma*-inoculated mice at 1, 2, 14, and 28 days, respectively, than in controls (all $P < 0.05$) (Fig. 5). There were few alveolar and interstitial inflammatory cells in untreated and broth-inoculated controls.

U. urealyticum pneumonia-mediated lung injury. Despite the influx of inflammatory cells, there were no differences in BAL

protein concentration during the first 28 days (Fig. 6), suggesting that pulmonary vascular endothelial barrier function remained intact. The histology in infected mice was marked by progressive focal loss of the airway ciliated epithelium and a mild increase in interstitial cellularity (Fig. 7). By 28 days postinoculation, marked multifocal squamous metaplasia of the airway epithelia of infected mice was observed. Focal peribronchiolar granulomas with macrophage predominance, increased bronchiolar-associated lymphoid tissue, and multifocal areas of mild emphysema were common features at 28 days postinoculation (Fig. 7C).

Cytokine response in U. urealyticum-infected lungs is modest and delayed. Analysis of BAL cytokine composition revealed moderate reductions in TNF-α (47%) at 3 days and MCP-1 (73%) at 7 days in *Ureaplasma*-inoculated mice ($P < 0.05$) (Fig. 8A and B). However, by 14 days postinoculation, there was a trend toward increased TNF-α, and by 28 days there was an increase in GM-CSF (Fig. 8C) and IL-10 (Fig. 8D) in the *Ureaplasma*-inoculated mice. There were no significant differences in MIP-2, KC, IL-6, or IL-11 between *Ureaplasma*-inoculated mice and either sham-inoculated or untreated controls (data not shown).

DISCUSSION

By serial adaptation of *U. urealyticum* to the C3H/HeN mouse strain, we have developed the first juvenile model of *Ureaplasma* pneumonia. The infection was characterized by three distinct phases: (i) acute inflammation (days 1 to 2), (ii) apparent resolution of acute inflammation (days 3 to 7), and (iii) chronic inflammation and mild lung injury (days 14 to 28). The first phase was characterized by an influx of PMN and macrophages in the airways and lung tissue without significant
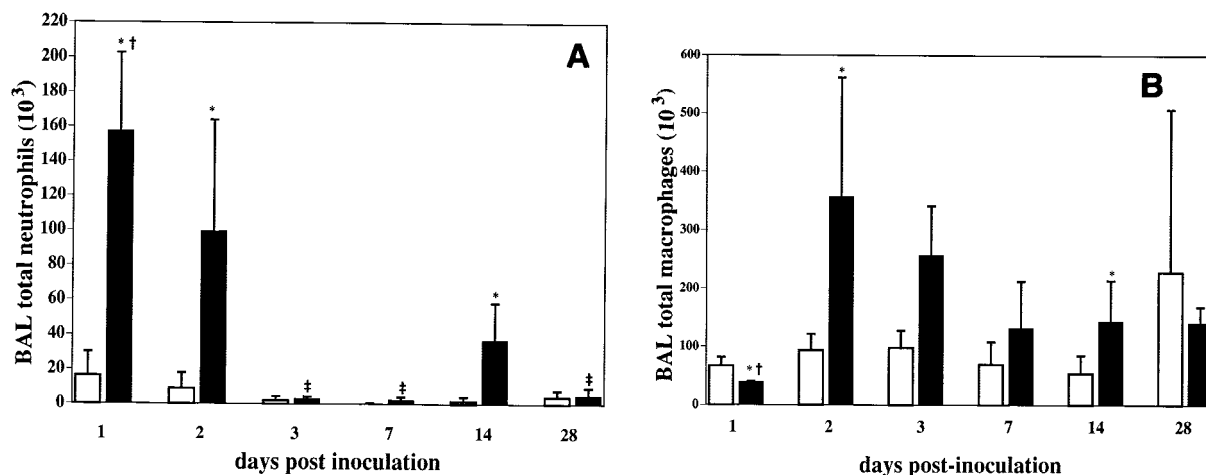
FIG. 3. BAL inflammatory cell composition after *U. urealyticum* inoculation. Lungs of sham-inoculated and *Ureaplasma*-inoculated animals were lavaged in situ with PBS. Total-cell counts were performed manually with a hemacytometer, and differential cell counts of Diff-Quick-stained cytopreparations were performed. Data are expressed as the means ± SE of the total number of neutrophils (A) and total number of macrophages (B). White bars, broth-inoculated controls; black bars, *Ureaplasma*-inoculated animals. *, $P < 0.05$ compared to controls inoculated with broth on the same day; †, $P < 0.05$ compared to *Ureaplasma* group at all other time points; ‡, $P < 0.05$ compared to day 14 *Ureaplasma* group.

elevations of cytokines or chemokines. During the second phase, levels of inflammatory cells in the *Ureaplasma*-inoculated lungs were similar to that of controls, and BAL concentrations of TNF-α and MCP-1 in infected mice were lower than those in control mice. Despite clearance of organisms by 14 days postinoculation, there were persistent abnormalities of the airway epithelium and chronic inflammation from 14 to 28 days postinoculation.

Differences in host factors and isolate virulence may affect the ability to adapt *Ureaplasma* to a nonhuman host. A newborn-mouse *Ureaplasma* pneumonia model demonstrated strain and age differences in susceptibility to infection. Rudd and coworkers (39) were able to infect C3H/HeN newborn mice with human newborn-derived, mouse-adapted serotypes 1 and 10 and, to a lesser extent, C57BL/6N newborn mice and 14-day-old mice of either strain. We also observed differences in mouse strain susceptibility and isolate virulence. Initial attempts to infect CD-1 outbred mice with the ATCC serotype 3 reference strain or the biovar 2 clinical isolate were unsuccessful. Only the C3H/HeN mice were successfully infected with the clinical isolate. Differences in the inoculation method may also account for differences in infection rates in the present study and the study of Rudd et al. (39). In the newborn mouse model, the mice were inoculated intranasally while awake. Since swallowing was not suppressed, the 2-week-old mice may have swallowed rather than aspirated the inoculum. In the present study swallowing was inhibited by deep anesthesia and the inoculum was directly aspirated.

The histopathologic findings of our juvenile-mouse model are similar to those observed in stillborn (13) and full-term human infants (6, 36, 45) and in a newborn primate model of *Ureaplasma* pneumonia (46). A hallmark feature in these studies is the focal loss of ciliated respiratory epithelium. Immunofluorescence of term infant lung infected with *Ureaplasma* serotype 8 (biovar 2) revealed adherence of organisms in fine clumps and strands along alveoli and terminal bronchioles (36). In a second case report of a *Ureaplasma*-infected stillborn

fetus, scanning electron microscopy of the trachea showed focal areas of decreased ciliated epithelial cells, with residual cilia being clumped or flattened (13). In the present study, epithelia of large and small airways were affected, with changes apparent as early as 24 h postinoculation and persisting throughout the period of study. We speculate that airway epithelial injury may follow adherence of *Ureaplasma* to the cells and utilization of host cell nutrients and local release of toxins such as ammonia, hydrogen peroxide, or other hemolysins (14). Inhibition of ciliary function has been proposed as a pathogenic mechanism for other mycoplasmas (7) and may increase host susceptibility to secondary infection due to decreased clearance of inhaled pathogens. The epithelial injury observed in the present study differs significantly from that due to murine *M. pulmonis* infection, of which hyperplasia of airway epithelium is characteristic (41). The observed airway injury is unlikely to be due to the inoculation method, since similar lesions were not observed in broth-inoculated control animals.

There was an inflammatory-cell influx in the lungs of *Ureaplasma*-inoculated mice. There was an early influx at 24 to 48 h characterized initially by an increase in neutrophils in the bronchoalveolar and interstitial compartments and subsequently by macrophages in both compartments. Increases in BAL and interstitial neutrophils and macrophages were again evident during the chronic phase (days 14 to 28). This is similar to histologic findings in experimental animal models of *U. urealyticum* infection (8, 39, 46) and in human *Ureaplasma*-infected preterm (43) and full-term (6, 36, 45) infants dying with acute pneumonia. Murine models of *Mycoplasma pneumoniae* demonstrated a different pattern of inflammation. In these models, there were more intense acute peribronchiolar, bronchiolar, and perivascular infiltrates that resolved by 3 to 4 weeks postinoculation (20, 29). In contrast, chronic naturally acquired *M. pulmonis* infection in rodents is characterized by peribronchial lymphoid hyperplasia (41).

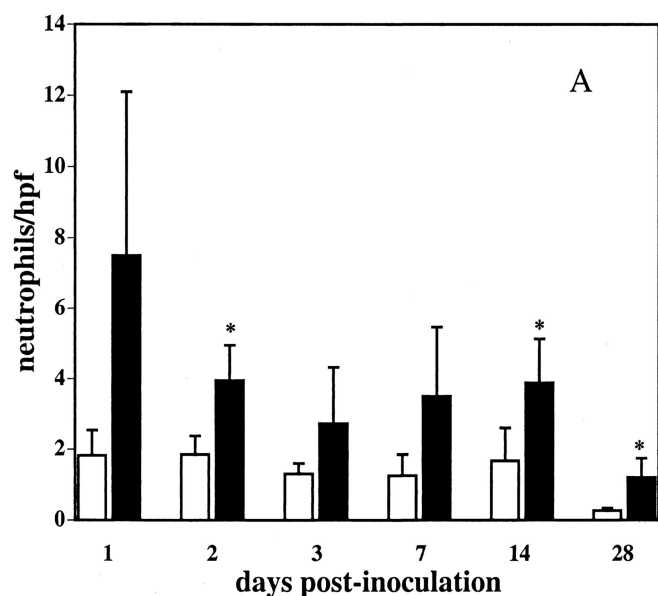In the present study, *U. urealyticum* inoculation of sponta-
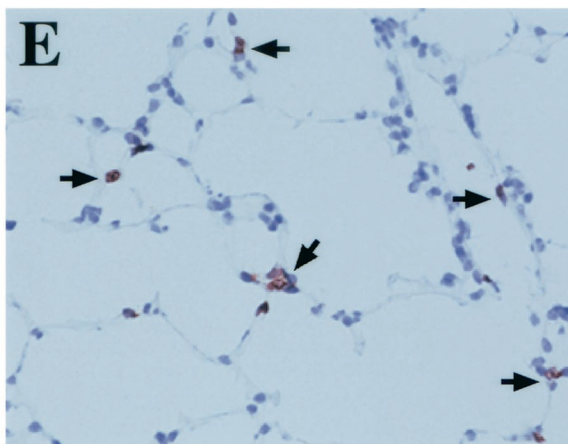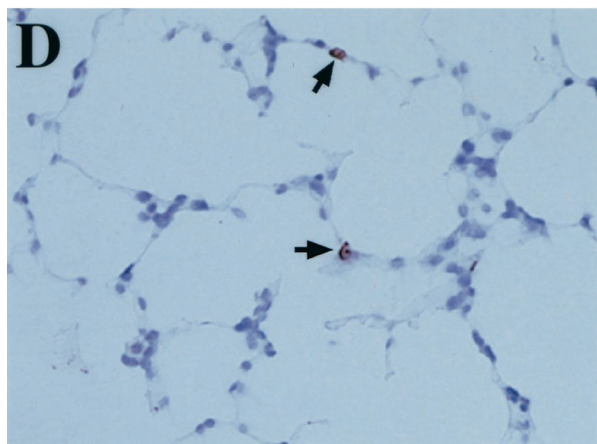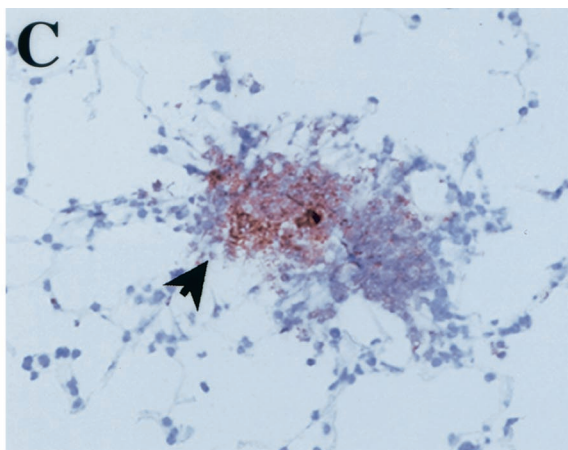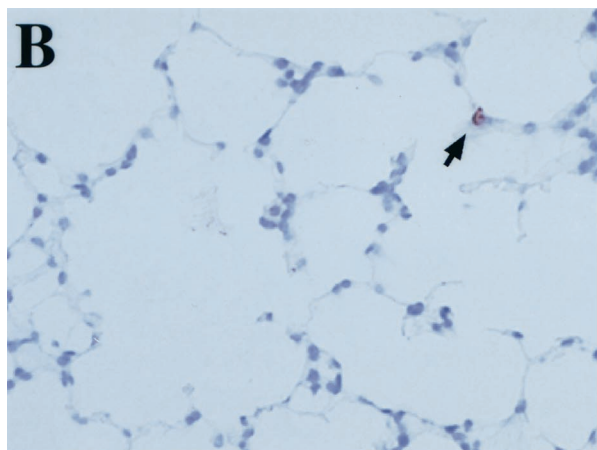
FIG. 4. Changes in the number of PMN immunoreactive cells in the lung after *U. urealyticum* inoculation. Cryosections (8 μm) of OCT-embedded, acetone-fixed lungs from *Ureaplasma*- and sham-inoculated mice were immunostained with biotinylated rat anti-mouse Gr-1 antibody and counterstained with hematoxylin. (A) Data are expressed as the means ± SE of the numbers of positively stained PMN per hpf in four fields from comparable regions in each slide. White bars, sham-inoculated controls; black bars, *Ureaplasma*-inoculated animals. (B to E) Representative immunostained sections of lungs 2 days postinoculation from a broth-inoculated control mouse (B) and a *Ureaplasma*-inoculated mouse (C) and 14 days postinoculation from a broth-inoculated control mouse (D) and a *Ureaplasma*-inoculated mouse (E). Arrows, immunoreactive PMN. ∗, $P < 0.05$ compared to controls inoculated with broth on the same day.

neously breathing mice did not stimulate significant increases in BAL cytokines or chemokines during the acute phase of infection. TNF-α concentrations were lower in *Ureaplasma*-infected animals at 3 days postinoculation but tended to be higher than control levels 14 days postinoculation, coinciding with an influx of PMN and macrophages. This time course suggests that *Ureaplasma* may interfere with the early innate immune response but may subsequently lead to chronic, dys-
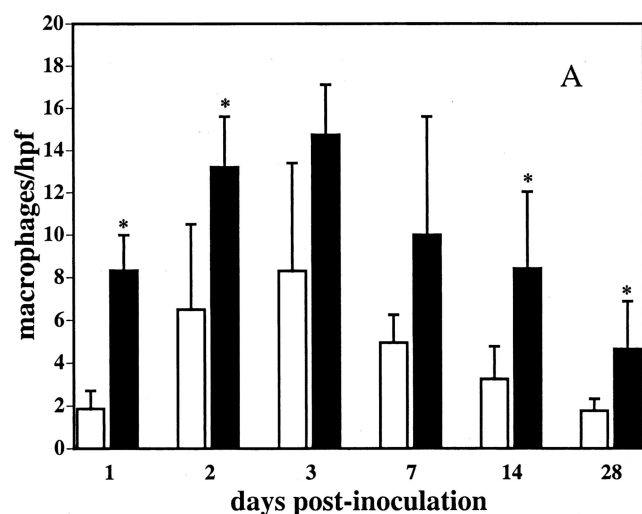
FIG. 5. Changes in the number of macrophage immunoreactive cells in the lung after *U. urealyticum* inoculation. Cryosections (8 μm) of OCT-embedded, acetone-fixed lungs from *Ureaplasma*- and sham-inoculated mice were immunostained with rat anti-mouse Mac-3 and counterstained with hematoxylin. (A) Data are expressed as the means ± SE of the numbers of positively stained macrophages per hpf in four fields from comparable regions in each slide. White bars, sham-inoculated controls; black bars, *Ureaplasma*-inoculated animals. (B to E) Representative immunostained sections of lungs 2 days postinoculation from a broth-inoculated control mouse (B) and a *Ureaplasma*-inoculated mouse (C) and 14 days postinoculation from a broth-inoculated control mouse (D) and a *Ureaplasma*-inoculated mouse (E). *, $P < 0.05$ compared to controls inoculated with broth on the same day.
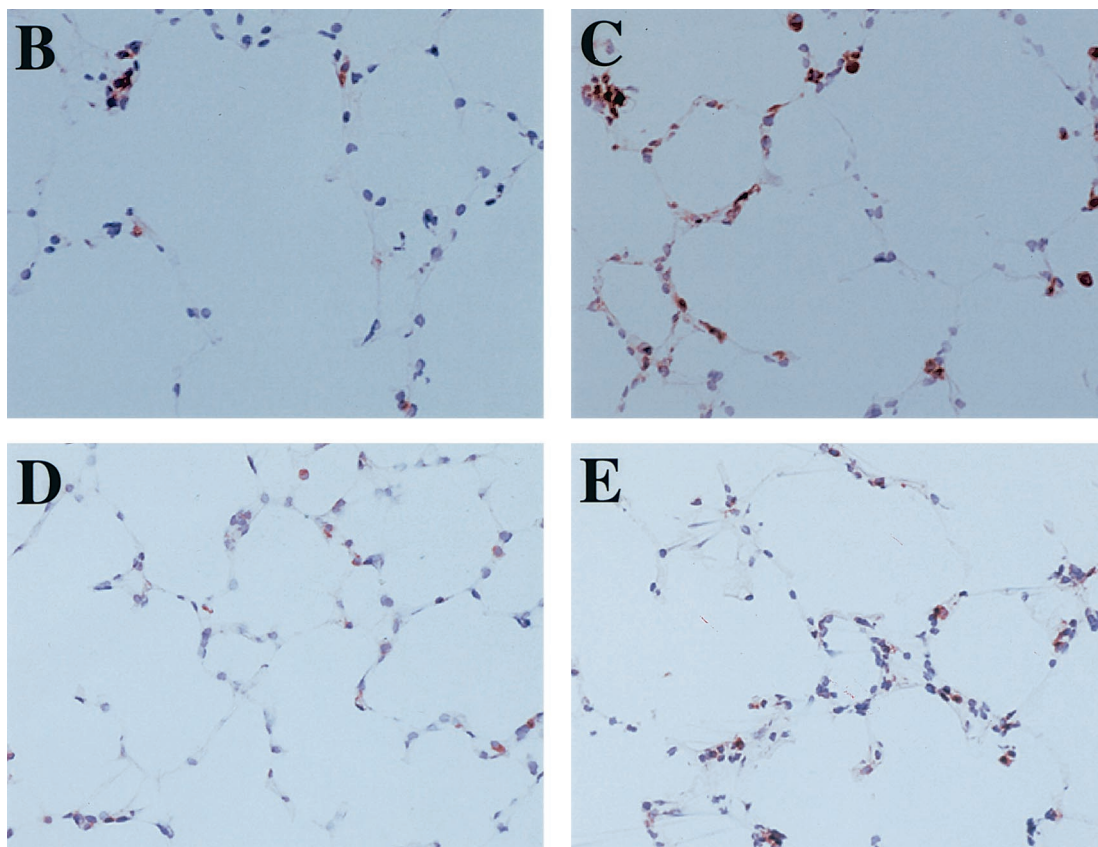
regulated inflammation. The contention that attenuating early proinflammatory events can lead to chronic inflammation is supported by studies showing that TNF-α-deficient mice mount an inflammatory response that is dysregulated and lethal rather than self-limited when challenged with heat-killed *Corynebacterium parvum* (28).

While pulmonary cytokine expression tends to increase during the chronic rather than the acute phase of *Ureaplasma* infection in juvenile mice, respiratory tract colonization with *U. urealyticum* in preterm intubated infants has been associated with increased tracheal aspirate concentrations of TNF-α (34), IL-1β (17, 18, 34), IL-8 (4, 18), and MCP-1 (4). However, many if not all affected infants are infected in utero so that day 1 of life may represent the chronic phase of infection. Immunohistochemical studies of autopsy lung specimens demonstrated that pulmonary macrophages in infants who died during the chronic phase of *Ureaplasma* pneumonia expressed TNF-α (43). Since all infants in these studies were intubated and mechanically ventilated with variable supplemental oxygen exposure, the effects of *Ureaplasma* alone cannot be distin-
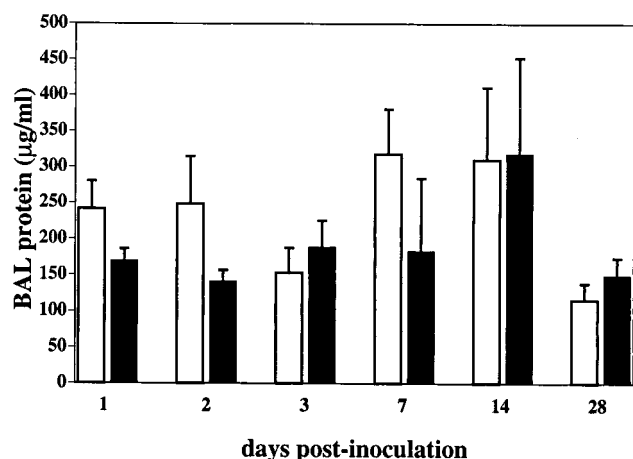
FIG. 6. Effect of *U. urealyticum* infection on BAL protein concentration during the month postinoculation. Lungs of sham-inoculated and *Ureaplasma*-inoculated animals were lavaged in situ with PBS. BAL protein was measured by the Bradford method (5). Data are expressed as means ± SE. White bars, sham-inoculated controls; black bars, *Ureaplasma*-inoculated mice.

guished from the combined effects of lung injury from barotrauma and/or hyperoxia. This is consistent with the hypothesis that *Ureaplasma* is a cofactor rather than a primary factor in neonatal lung injury.

In vitro studies demonstrated that high inocula of *Ureaplasma* ($\geq 10^6$ CCU/ml) stimulated TNF-α release by preterm cord blood monocytes (27), RAW 264.7, a murine macrophage cell line (9), THP-1, a human monocytic cell line, NR 8383, a rat alveolar macrophage cell line, and human lung macrophages isolated from tracheal aspirates from preterm infants (26). However, *Ureaplasma* serotype 3 (*U. parvum*, biovar 1) at low inoculum (10^3 CCU), which might be more representative of human lung colonization, stimulated only low levels of TNF-α release by cultured preterm monocytes but greatly augmented LPS-induced TNF-α release by the same cells (27). These data suggest that *Ureaplasma* infection in juvenile mice and human neonates causes little acute inflammation but that chronic airspace inflammation develops and is characterized by PMN and macrophage recruitment, which might alter the response to subsequently inhaled pathogens or other inflammatory stimuli.
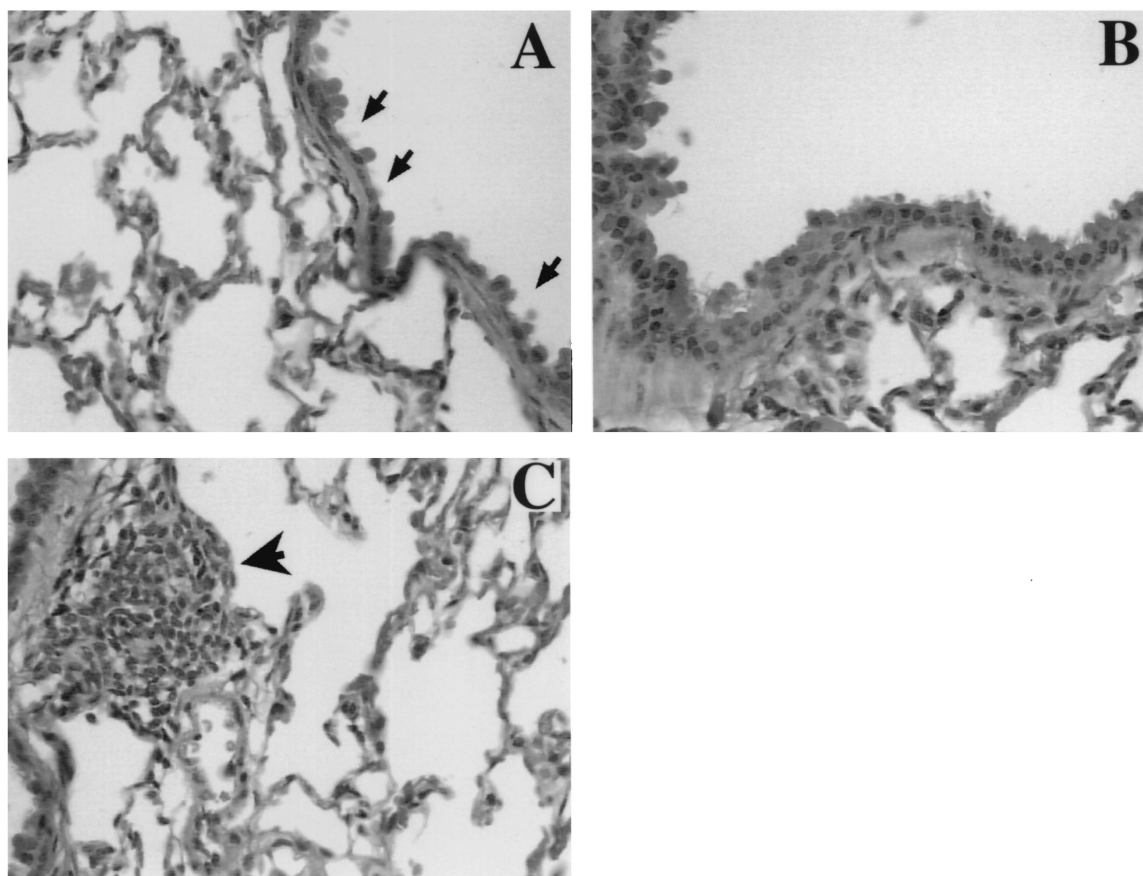

FIG. 7. Histopathologic appearance of lungs of *U. urealyticum*-inoculated mice 1 month postinoculation. Hematoxylin-stained sections of formalin-fixed paraffin-embedded lungs from sham-inoculated and *Ureaplasma*-inoculated mice were analyzed. There was focal loss of airway ciliated epithelia in *Ureaplasma*-inoculated mice (A, arrows) compared to preserved normal epithelia in sham-inoculated controls (B). Focal peribronchial granulomas were evident 28 days postinoculation in *Ureaplasma*-inoculated mice (C, arrowhead).
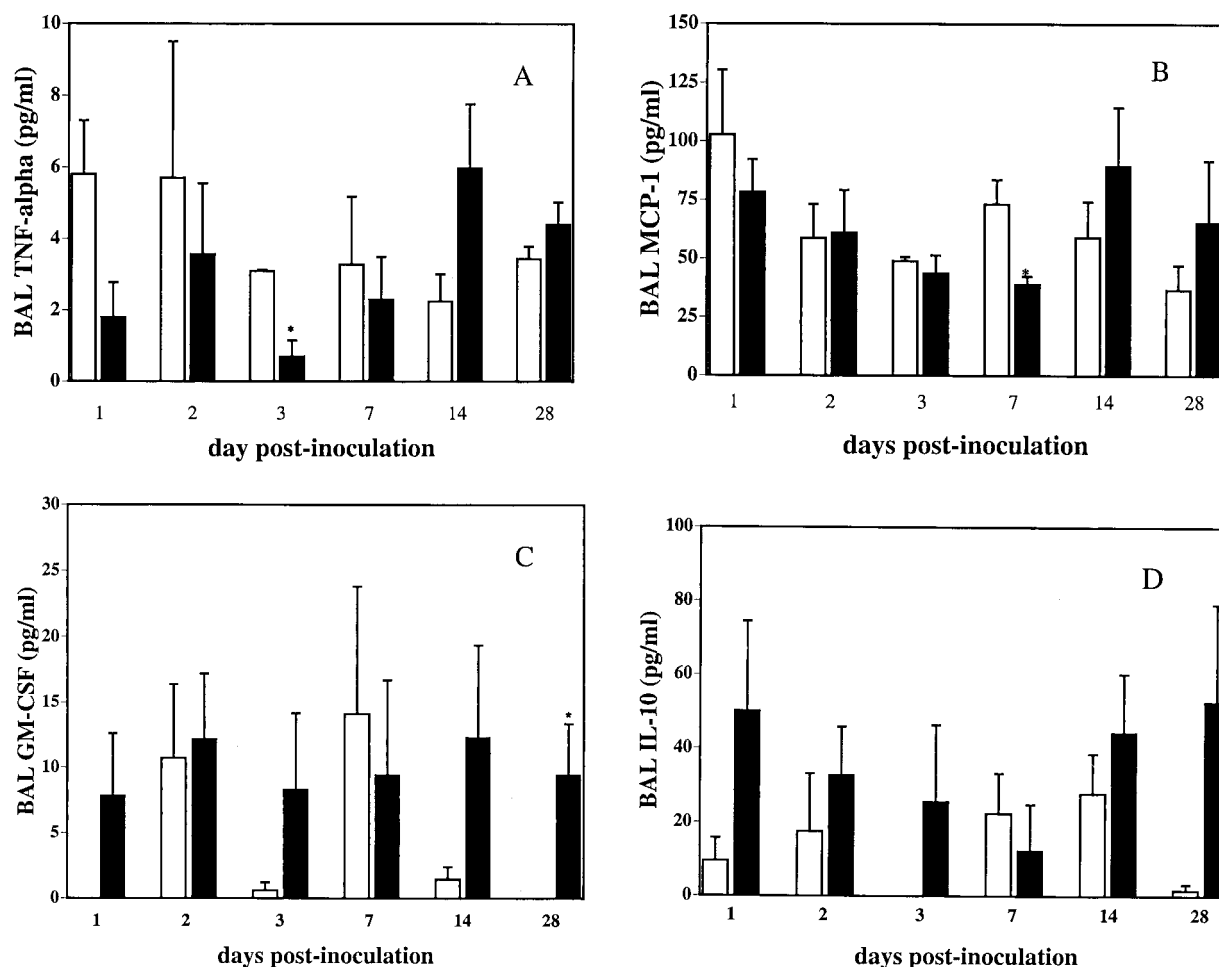
FIG. 8. Effect of *U. urealyticum* inoculation on BAL cytokine concentrations. Cytokines in BAL were measured by using standard two-antibody ELISA with commercial antibody pairs and recombinant standards. (A) TNF-α; (B) MCP-1; (C) GM-CSF; (D) IL-10. Data are expressed as means ± SE. White bars, sham-inoculated controls; black bars, *Ureaplasma*-inoculated mice. ∗, *P* < 0.05 compared to controls inoculated on the same day.

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